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The endocrine disrupting potential of monosodium glutamate (MSG) on secretion of the glucagon-like peptide-1 (GLP-1) gut hormone and GLP-1 receptor interaction

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Abstract

Monosodium glutamate (MSG) is a suspected obesogen with epidemiological evidence positively correlating consumption to increased body mass index and higher prevalence of metabolic syndrome.

ELISA and high content analysis (HCA) were employed to examine the disruptive effects of MSG on the secretion of enteroendocrine hormone glucagon-like peptide-1 (GLP-1) and GLP-1 receptor (GLP-1R), respectively. Following 3 h MSG exposure of the enteroendocrine pGIP/neo: STC-1 cell line model (500 µg/ml) significantly increased GLP-1 secretion (1.8 fold; $P<0.001$), however, 72 h exposure (500 µg/ml) caused a 1.8 fold decline ($P<0.05$). Also, 3 h MSG exposure (0.5-500 µg/ml) did not induce any cytotoxicity (including multiple pre-lethal markers) but 72 h exposure at 250-500 µg/ml, decreased cell number (11.8-26.7%; $P<0.05$), increased nuclear area (23.9-29.8%; $P<0.001$) and decreased mitochondrial membrane potential (13-21.6%; $P<0.05$). At 500 µg/ml, MSG increased mitochondrial mass by 16.3% ($P<0.01$). MSG did not agonise or antagonise internalization of the GLP-1R expressed recombinantly in U2OS cells, following GLP-1 stimulation. In conclusion, 72 h exposure of an enteroendocrine cell line at dietary levels of MSG, results in pre-lethal cytotoxicity and decline in GLP-1 secretion. These adverse events may play a role in the pathogenesis of obesity as outlined in the *obesogen hypothesis* by impairing GLP-1 secretion, related satiety responses and glucose-stimulated insulin release.

Key terms: Monosodium glutamate, *in vitro* bioassays, high content analysis, GLP-1, diabetes; obesity

1. Introduction

The incidence of obesity and metabolic diseases such as diabetes has risen dramatically over the past two decades. The United Kingdom (UK) is officially the most obese country in Europe with 1 in 5 adults overweight and 1 in 15 adults obese. It is predicted that the number of obese individuals will soar by a staggering 73% to 26 million people in the UK over the next twenty years. This is expected to fuel increases in cases of diabetes which in 2010 were estimated at 285 million worldwide with a predicted increase by 54% in 2030 due to population growth, ageing of populations and urbanization with associated lifestyle change (Shaw *et al.*, 2010). Increased caloric intake and decreased physical activity are undoubtedly the major drivers of these increases but other factors have been highlighted (COT, 2013). Several lines of evidence, including several human epidemiological studies, suggest that certain chemicals in the diet or environment (referred to as ‘obesogens’) play a role in promoting obesity (Sharpe and Drake, 2013).

Monosodium glutamate (MSG) is a flavour enhancer used in many household prepared and processed foods worldwide which has recently been highlighted as a suspected dietary obesogen (Holtcamp, 2012). It is also believed that the effects of MSG are compounded further by its widespread availability in manufactured foods and the high frequency with which some individuals consume it (Mercola, 2009). A study investigating the effect of a lipid-containing meal with added MSG (2000 mg/meal) on glucose homeostasis, incretin secretion and gastric emptying in humans demonstrated GLP-1 plasma levels were significantly increased (Hosaka *et al.*, 2012).. This demonstrates that exposure to MSG can have effects on the regulation of gut hormone secretion and consequently, glucose and weight homeostasis and highlights its potential to act as an endocrine disruptor.

The reported daily MSG intake of individuals varies greatly across the population. The reported UK average intake in 1991 was 580 mg/day for the general population but 4.68 g/day for excessive consumers. In 2000, the average intake ranged from 300-1000 mg/day in industrialised countries (Husarova and Ostatnikova, 2013). However, more recent studies state that the average intake of MSG is around 0.4 g/day in Europe and has been steadily increasing (Collision *et al.*, 2009, He *et al.*, 2011, Nakanishi *et al.*, 2008). It is also important to consider Asian countries, where MSG consumption is much higher than other parts of the world. A study conducted in China found that the mean intake of MSG for the entire population was 3.1 g/ day (Shi *et al.*, 2014). Current legislation does not impose any limit on the amount of MSG that restaurants or the food industry can add to their products. Furthermore, food processors and manufacturers are not obligated to list the amount of MSG

on their packaging. This anomaly makes it difficult to accurately monitor levels of MSG consumption and underestimations most likely occur.

A number of studies have investigated potential links between MSG, obesity and other metabolic disorders such as diabetes. In a longitudinal study, He *et al.* (2011) examined the association between MSG consumption and the incidence of overweight adults, reporting that a cumulative mean (\pm standard deviation) MSG intake of 2200 ± 1600 mg/day is associated with an increase in body mass index (BMI) after adjustment for potential confounders. The extent of weight gain in MSG users compared with non-users was modest. However, there is still major public health interest in knowing whether weight gain is modulated by MSG intake. Further studies are necessary to investigate potential mechanisms where MSG could enhance the risk of obesity/diabetes (He *et al.*, 2011). A study conducted in Thailand reported a significantly higher prevalence of metabolic syndrome in the tertile with the highest MSG intake. Furthermore, for every 1000 mg more MSG intake per day, the risk of having metabolic syndrome or being overweight increased, regardless of the individual's total energy intake or physical activity level (Insawang *et al.* 2012).

Epidemiological studies and animal studies demonstrate an association between MSG consumption/MSG-injected models and weight gain (Clough *et al.*, 1986; He *et al.*, 2008; Hirata *et al.*, 1997; Insawang *et al.*, 2012; Iwase *et al.*, 1998; Olney, 1969; Svidnicki *et al.*, 2013). Miskowiak *et al.* (1993) found that rats administered with MSG displayed abnormal growth, obesity and reduced mass of the pituitary glands and testes, and also lower testosterone levels.

Most known or suspected obesogens are endocrine disruptors (EDs). Many are widespread with exposure suspected or confirmed as quite common (Holtcamp, 2012). Metabolic disorders may be influenced by endocrine disrupting interactions between consumed MSG and the hormones involved in controlling satiety responses and insulin release. Glucagon-like peptide 1 (GLP-1) is an intestinal gut hormone with important physiological roles including appetite control and is key in the regulation of post-prandial increases in insulin secretion, thereby regulating levels of glycaemia (Baggio and Drucker, 2007; Drucker, 2006). GLP-1 mediates its effects through the GLP-1 receptor (GLP-1R) which is a G-protein coupled receptor (GPCR) expressed in a wide range of human tissues including α , β and δ - cells of the pancreatic islets, lung, kidney, heart, intestine, stomach, skin, vagal nerve and several regions of the central nervous system (CNS) including the hypothalamus and brainstem. GLP-1Rs

have been shown to rapidly internalise following activation by GLP. A study by Kuna et al., 2013 demonstrated that GLP-1 receptor internalisation was induced by 0.1 μ M GLP-1 2 minutes to 1 h. Internalised receptors are then either recycled back to the cell surface where they can again engage with ligands, or are targeted for post-endocytic degradation, which results in permanent signal termination from the receptor (Noerklit et al., 2014).

GLP-1 is well established to regulate bodyweight by stimulating feelings of satiety via GLP-1R in the brain (Baggio and Drucker, 2007; Drucker, 2006). In addition to the most well characterised effect, the amplification of glucose-stimulated insulin secretion, GLP-1 is now known to induce expansion of insulin-secreting β -cell mass. The mechanism by which GLP-1 is believed to enhance insulin secretion is through the regulation of ion channels (including ATP-sensitive K^+ channels, voltage-dependent Ca^{2+} channels, voltage-dependent K^+ channels, and nonselective cation channels) and by the regulation of intracellular energy homeostasis and exocytosis (Macdonald et al., 2002). A number of clinical trials have been conducted in order to investigate the weight loss effect of GLP-1R agonist therapies in diabetic patients. A correlation between these therapies and weight loss has been established with the GLP-1R agonist liraglutide having a significant positive effect on weight loss (Buse et al., 2013).

Hosaka et al., (2012) investigated the effect of a lipid-containing meal with added MSG (2000 mg/meal) on glucose homeostasis, incretin secretion and gastric emptying in humans. GLP-1 plasma levels were significantly increased (although the mechanism for this was not determined). One possible mechanism could be through the interaction of the GLP-1R. It is well-established that sustained GLP-1R antagonism (with exendin (9-39)) impairs glucose tolerance, decreases insulin secretion and causes hyperglycaemia (D'Alessio et al., 1996; Edwards et al., 1999; Kolligs, Fehmann et al., 2002; Green et al., 2005; Scrocchi et al., 1996). As MSG is proposed to be an obesogen it seems logical to investigate whether it can disrupt the GLP-1/GLP-1R axis.

This is the first study assessing a potential obesogenic mechanism for MSG and the aims are to primarily investigate 3 h and 72 h exposure effects of MSG on GLP-1 secretion *in vitro* and also to investigate any potential mechanisms through 1) examining 3 h and 72 h toxicity of MSG on enteroendocrine (EE) cells (pGIP/neo: STC-1 cells, a sub-clone of the STC-1 cell line which secretes GLP-1) including measures of subtle pre-lethal cytotoxicity and 2)

studying the endocrine disrupting potential of MSG on the GLP-1 receptor using a GLP-1R redistribution assay and high content analysis (HCA).

2. Materials and methods

2.1. Chemicals and reagents

Cell culture reagents and Hank's Balanced Salt Solution (HBSS) were supplied by Life Technologies (Paisley, UK). The reference standard GLP-1 (7-37) was obtained from Abcam (Cambridge, UK). MSG, thiazolyl blue tetrazolium bromide (MTT) and formalin were all supplied by Sigma-Aldrich (Poole, Dorset, UK). Hoechst nuclear stain and mitochondrial membrane potential dye were provided by Thermo Scientific (UK). GLP-1 ELISA kits were purchased from Millipore (Billerica, MA, USA). All other reagents were standard laboratory grade.

2.2. Cell culture

The U2OS-GLP1R-EGFP cell line (a U2OS cell line with stable expression of the human GLP-1R with a C-terminal EGFP tag) was obtained from Thermo Scientific (UK) and grown in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO₂ and 95% humidity. The cells were cultured routinely in Dulbecco's modified Eagle medium (DMEM) media with Glutamax, 10% foetal bovine serum (FBS), 1% penicillin and streptomycin (50 U/ml) and 50 mg/ml G418. Cells were grown in plating media (DMEM with Glutamax, 1% FBS, 1% penicillin and streptomycin (50 U/ml)) for 24 h prior to running the assay. Test compounds and standards were diluted in assay media (DMEM with Glutamax, 1% penicillin and streptomycin (50 U/ml)). The assay has been validated with cells up to a passage of 30 (Thermo Scientific, UK).

pGIP/neo: STC-1 cells are an enteroendocrine (EE) cell model and its GLP-1 secretory ability has been extensively investigated (Gillespie *et al.*, 2015; Jafri *et al.*, 2016). These were a gift from Dr. B. Wice (Washington University of St. Louis) with permission from Dr. D. Hanahan (University of California, San Francisco, CA). Cells were cultured DMEM with Glutamax, 10% FBS, 1% penicillin and streptomycin (50 U/ml) and 50 mg/ml G418. Cells were passaged at 80-90% confluence and used between passages 15-50.

178

179 2.3. Cell viability

180 In addition to visual inspection of cells under the microscope to evaluate cell morphology and
181 attachment, two cell viability assays were used to assess cytotoxic effects of MSG exposure.

182 2.3.1. MTT

183 The thiazolyl blue tetrazolium bromide (MTT) assay was used to monitor the cytotoxic
184 effects of MSG test concentrations in the U2OS-GLP1R-EGFP cell line. Viable cells convert
185 the soluble yellow MTT into insoluble purple formazan by the action of mitochondrial
186 succinate dehydrogenase. For the U2OS-GLP1R-EGFP cells, clear flat-bottomed 96-well
187 plates (Nunc, Roskilde, Denmark) were seeded with 6×10^4 cells and the test compound was
188 added after 24 h and incubated for a further 1 h. The supernatant was discarded and 50 μ l of
189 MTT solution/well (5mg/ml stock in phosphate buffered saline (PBS) diluted in 1:2.5 in
190 assay media) was added and cells were incubated for a further 3 h. Again, supernatant was
191 removed and 200 μ l of DMSO was added to each well (to dissolve the formazan crystals) and
192 incubated for a further 10 min with agitation at 37 °C. Optical density was measured using a
193 Sunrise spectrophotometer at 570 nm with a reference filter at 630 nm (TECAN,
194 Switzerland). Samples were tested in triplicate and in three independent exposures. Viability
195 was calculated as a percentage absorbance of the sample when compared with the absorbance
196 of the solvent control.

197

198 2.3.2. Alamar Blue

199 The viability of the pGIP/neo: STC-1 cells in the acute and chronic study was determined
200 using the AlamarBlue®. After removal of media for hormone analysis, 1 ml of 1:10 (v:v)
201 AlamarBlue® in cell culture medium was added to each well and incubated for 24 h. A 100
202 μ l volume was then removed from each well and added into clear flat-bottomed 96-well
203 microtiter plates (BD Biosciences, Bedford, MA, US). Using a Sunrise spectrophotometer
204 (TECAN, Switzerland) the absorbance was measured at 570 nm and 600 nm. Viability was
205 calculated as the percentage absorbance of the sample in comparison with the absorbance of
206 the solvent control (0.1%, v:v dH₂O (deionised water) in media).

207

2.4. GLP-1R redistribution assay

U2OS-GLP1R-EGFP cells were seeded (100 μ l of 6×10^4 cells/well) in 96-well black plates with clear, flat bottoms. The cells were allowed to attach for 24 h before adding 100 μ l of the test sample (MSG) and the GLP-1 (7-37) standards at a final dH₂O and dimethyl sulfoxide (DMSO) concentration of 0.2%. To assess whether MSG had a disruptive (or antagonistic) effect on the interaction of GLP-1 with the GLP-1R, the potential of MSG to influence GLP-1-mediated internalisation of the EGFP-tagged GLP-1R was assessed. Thus, the ability of 150 nM GLP-1 to induce internalisation of the receptor was assessed in the absence and presence of MSG. Cells were incubated for 1 h in a 37 °C, 5% CO₂, humidified incubator, before decanting buffer and fixation of the cells with 100 μ l/well of formalin (approximately 4% formaldehyde). Cells were incubated for a further 20 min at room temperature and then washed four times with 200 μ l PBS per well per wash followed by addition of 100 μ l of 1 μ M Hoechst staining solution (1 μ l: 10 ml PBS) to each well. After 30 min, the plate was imaged on a Thermo Scientific Arrayscan HCS. The filters were set for the Hoechst dye (350/461 nm) and GFP/FITC (488/509 nm). The SpotDetectorV3 BioApplication was used for carrying out the reading step of the GLP-1R redistribution assay using the ObjectSpotAvgIntensity parameter and both data and corresponding images are generated (see Fig 1 for example of images). The assay was performed in triplicate for each experimental point and repeated in three independent exposures. The response of the cell line to the various compounds was measured and compared with the dH₂O solvent control.

2.5. HCA cytotoxicity assay

The Cellomics® HCS reagent series multiparameter cytotoxicity assay was performed according to the manufacturer's instructions. pGIP/neo: STC-1 cells were seeded 6×10^4 in 96 well plates 24 hours prior to the assay. Briefly, mitochondrial membrane potential dye was prepared by adding 117 μ l of anhydrous DMSO to make a 1 mM stock. Following incubation (3 h or 72 h), 50 μ l of live cell stain was added to each well for 30 min at 37 °C and protected from light. The live stain was removed and cells were then fixed with a 10% formalin solution for 20 min at room temperature, protected from light and washed with PBS. Hoechst 33342 dye at a final concentration of 1.6 μ M was added to each well and incubated for 10 min at room temperature and protected from light; after which cells were washed with PBS four times and evaluated on a CellInsight™ NXT High Content Screening (HCS) Platform

(Thermo Fisher Scientific, UK). Hoechst stain was used to measure cell number and nuclear morphology including nuclear intensity and nuclear area while mitochondrial membrane potential dye was used to measure mitochondrial health, specifically mitochondrial membrane potential and mitochondrial mass. Data were captured for each plate using a $\times 10$ objective magnification in the selected excitation and emission wavelengths of Hoechst dye (Ex/Em 350/461 nm) and mitochondrial membrane potential dye (Ex/Em 554/576 nm). For each well, 25 field of view images were acquired to examine each parameter with each image containing approximately 6,000 cells.

2.6. *GLP-1 secretion studies*

2.6.1. *3 h exposure*

The pGIP/neo: STC-1 cells were seeded into 24-well plates (1×10^6 per well) and cultured overnight at 37 °C in a humidified atmosphere of 5% CO₂. Medium was removed and cells were washed once with HBSS buffer and equilibrated for 1 h in 500 µl HBSS buffer. Test compounds (0.5, 5, 50, 250, 500 µg/ml MSG in dH₂O) and dH₂O solvent control diluted in HBSS buffer (1000 µl) were added and allowed to incubate for a further 3 h. Supernatant was removed and stored at -20°C prior to hormone analysis.

2.6.2. *72 h exposure*

Initially cells were seeded into 96 well plates (5×10^4 per well) and cultured overnight at 37 °C in a humidified atmosphere of 5% CO₂. Test concentrations (0.5, 5, 50, 250, 500 µg/ml MSG in dH₂O) and dH₂O solvent control diluted in reduced serum media (1% serum) (100 µl) were added and allowed to incubate for 72 h. Media from each well was harvested and retained at 24 h intervals and replaced by fresh media containing the appropriate concentration of MSG or vehicle control. Samples were stored at -20 °C until immediately before assay of the GLP-1 content at which time the 3 x 200 µl samples from each well were pooled.

2.7. *Hormone Analysis*

GLP-1 was measured using a Glucagon Like Peptide-1 (GLP-1) (Active) ELISA kit (EGLP-35K; in accordance with the manufacturer's instructions (Millipore, Watford, UK). All experiments were performed in triplicate for each experimental point and repeated in three independent experiments. Responses of cells to the test compound were compared with the negative control (dH₂O, 0.1%).

2.8. Statistical Analysis

All values shown are expressed as mean \pm standard error of the mean (SEM) of the three independent exposures for MSG. Data from the cell viability, GLP-1R redistribution assay and GLP-1 ELISA were analysed using Microsoft Excel and GraphPad PRISM 5 software (GraphPad Software Inc, San Diego, CA). A one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to determine significant differences between treatments and the corresponding control. A P-value of < 0.05 was considered as significant ($P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***).

3. Results

3.1. Cell viability data

Viability of the pGIP/neo: STC-1 cells was not affected by any concentration of MSG during either the acute or the chronic study, as determined by AlamarBlue® assay ($P > 0.05$) (data not shown). Although not significantly different, there was some evidence of reduced viability at higher concentrations of MSG at 72 h. However as this is a relatively insensitive assay there was sufficient justification for looking at potential cytotoxic markers on HCA.

Similarly, MTT assays showed no significant change in viability of the U2OS-GLP1R-EGFP cells following 1 h exposure to MSG (0.5, 5, 50, 250 and 500 $\mu\text{g/ml}$) (data not shown).

3.2. Effects of MSG on GLP-1 secretion

3.2.1. Effects of 3 h exposure of MSG on GLP-1 secretion

Following 3 h exposure of pGIP/neo: STC-1 cells to lower MSG concentrations (0.5-250 $\mu\text{g/ml}$) did not significantly affect GLP-1 secretion. However, at 500 $\mu\text{g/ml}$, MSG stimulated

a significant 9.6 pg /10⁶ increase in GLP-1 secretion (22.4 pg /10⁶ cells in 500 µg/ml MSG vs. 12.8 pg /10⁶ cells in control; $P < 0.001$) (Fig.2).

3.2.2. Effects of 72 h exposure of MSG on GLP-1 secretion

Similarly 72 h exposure of pGIP/neo: STC-1 cells to lower MSG concentrations (0.5-250 µg/ml) did not significantly affect GLP-1 secretion. However, at 500 µg/ml MSG there was a significant decrease in GLP-1 secretion by 35.9 pg/10⁶ cells (45.4 pg /10⁶ cells in 500 µg/ml MSG vs 81.4 pg /10⁶ cells in control $P < 0.05$) (Fig.3).

3.3. Cytotoxicity

The potential cytotoxicity of either 3 h or 72 h exposure to MSG (0.5, 5, 50, 250 and 500 µg/ml) on pGIP/neo: STC-1 intestinal cells was assessed by HCA analysis by measuring cell number and nuclear morphology including nuclear intensity, and nuclear area.

Mitochondrial membrane potential dye was used to measure mitochondrial health, specifically mitochondrial membrane potential and mitochondrial mass. For 3 h MSG exposure no cytotoxicity was observed at any MSG concentrations (data not shown).

However, following 72 h exposure, 250 µg/ml MSG decreased cell number by 11.8% ($P < 0.05$), increased nuclear area by 23.9% ($P < 0.001$) and decreased mitochondrial membrane potential by 13% ($P < 0.05$). MSG (500 µg/ml) decreased cell number by 26.7% ($P < 0.001$), increased nuclear area by 29.8% ($P < 0.001$), increased mitochondrial mass by 16.3% ($P < 0.01$) and decreased mitochondrial membrane potential by 21.6% ($P < 0.001$) (Fig. 4 and 5).

3.4. GLP-1R internalisation assay

A standard curve was generated using various concentrations of GLP-1 (1-300 nM) to drive receptor internalisation (Fig.6). The EC₅₀ value was calculated as 37 nM GLP-1.

MSG did not cause internalization of the GLP-1R in U2OS-GLP1R-EGFP cells. Furthermore, exposure to MSG (0.5-500 µg/ml) during the period of exposure to GLP-1 did not affect GLP-1-mediated internalisation of the GLP-1R (Figure 7).

325

326 **4. Discussion**

327 Associations between MSG consumption and increased incidence of diabetes and obesity
328 have been reported in both human and animal studies (He *et al.*, 2011; Insawang *et al.* 2012;
329 Miskowiak *et al.*, 1993). However, a potential mechanism has not yet been identified. This
330 study hypothesised that MSG might interact with the GLP-1 hormone, either through the
331 GLP-1R or by affecting GLP-1 secretion. For an obesogen, GLP-1 is a plausible potential
332 target given its pleiotropic actions on satiety responses, post-prandial insulin and glucagon
333 secretion, gut motility, and gastric acid secretion. The present study investigated whether
334 MSG has a disrupting effect on the secretion of the incretin hormone GLP-1 or its interaction
335 with its receptor. Cytotoxic responses were evaluated in pGIP/neo: STC-1 cells, an EE cell
336 model which secretes GLP-1. EE cells are one of four subtypes of epithelial cells lining the
337 gut (Beucher *et al.*, 2012) and collectively are the largest endocrine system in the body. The
338 cells used in this study (pGIP/neo: STC-1) are a sub-clone of the STC-1 cell line which
339 secretes GLP-1 (Gillespie *et al.*, 2015; Jafri *et al.*, 2016). Recent studies suggest that EE cells
340 are more flexible than previously thought because they alter the profile of hormones made
341 and released in response to nutrient changes in the diet (Habib *et al.* 2012). As pGIP/neo:
342 STC-1 cells are a pluri-hormonal EE cell line (also secreting cholecystokinin, peptide YY and
343 glucose-dependent insulinitropic polypeptide) (Hand *et al.*, 2012) that imitate some of the
344 other hormonal influences which exist in the intestine *in vivo* such as having the potential to
345 release other gut hormones.

346 A previous study on the STC-1 parental cells provides evidence that exposure to MSG can
347 activate the nutrient sensing T1R1/T1R3 (taste receptors) umami receptor. Additionally,
348 MSG exposure to the STC-1 cells induced neurotensin (NT) release, a peptide involved in the
349 regulation of hormone release from the gut (Kendig *et al.*, 2015). However, in the present
350 study the potential of MSG to interfere with GLP-1 interaction with the GLP-1R was
351 examined and under all of the various test conditions there was no evidence of this. It is
352 important to mention that the cell lines used for the present study and the Kendig *et al.*,
353 (2015) study differ, as well as the receptor investigated, therefore accounting for differences
354 in findings.

355 When studying the effects of MSG on intestinal EE cells, which produce and release GLP-1,
356 3 h exposure of MSG stimulated GLP-1 secretion with a concentration 500 µg/ml almost

doubling the amount of hormone that is released. Previously, it has been demonstrated *in vivo* that an acute bolus of MSG increases plasma GLP-1 in healthy male volunteers (Hosaka *et al.* 2012). The present data suggest that this could be a consequence of a direct effect of MSG on GLP-1 secretion by EE cells.

Secretion of GLP-1 occurs primarily in response to nutrient ingestion, particularly glucose and fat and to a lesser extent specific amino acids and protein hydrolysates (Elliott *et al.*, 1993, Ramshur *et al.*, 2002). However, it may not solely be the presence of nutrients that stimulates secretion. A biphasic release of GLP-1 into the circulation following a meal has been established (Rask *et al.*, 2001) which would suggest the potential input of neural and endocrine factors. It is believed that GLP-1 secretion may prove even more complex than other gut hormones. In particular, glutamine, a breakdown product of MSG, may be even more effective than glucose or other amino acids as a GLP-1 secretagogue. Glutamine triggers membrane depolarisation and increases intracellular calcium; however asparagine and alanine also increase intracellular calcium without increasing GLP-1 secretion (Reimann *et al.*, 2004). In the presence of diazoxide (a potassium channel activator) and a depolarising concentration of potassium chloride, glutamine still enhanced GLP-1 secretion (Reimann *et al.*, 2004), therefore glutamine-induced GLP-1 secretion may not be exclusively dependent on membrane polarisation. Therefore, the mechanism by which MSG induced an increase in GLP-1 secretion following 3 h exposure, may have been consequential of the breakdown product of MSG, glutamine. The paracrine actions of somatostatin, secreted from neighbouring D-cells exerts an inhibiting effect on GLP-1 secretion and may prove pivotal in producing a feedback loop for GLP-1 regulation following nutrient ingestion (Hansen *et al.*, 2000; Lahlou *et al.*, 2004). Previous studies using the parental STC-1 cell line have demonstrated that exposure to glutamine elevated the intracellular Ca^{2+} levels (Miyata *et al.*, 2014). It may be via this mechanism that the GLP-1 secretion is increased following 3 hour exposure to higher concentrations of MSG

MSG is consumed on a daily basis and is present in a wide variety of foods and therefore it was logical to examine the effects of a longer exposure on GLP-1 secretion (72 h) mimicking daily consumption. When pGIP/neo STC-1 cells were exposed to MSG for 72 h, a detrimental effect on GLP-1 secretion leading to a 1.8-fold reduction in the amount of hormone released was observed. A possible explanation for the chronic decrease in GLP-1 secretion is that MSG-induced stimulation of GLP-1 secretion (as observed with 3 h exposure) leads to a depletion in the capacity of EE cells to secrete GLP-1. However this

would need to be studied in more detail with further investigations. This observation has potential physiological relevance given that GLP-1 secretion may be blunted/impaired in patients with type 2 diabetes. For example, reduced postprandial concentrations of intact biologically active GLP-1 have been observed in type 2 diabetic patients and it seems likely that these reductions in GLP-1 secretion result in impaired insulin secretion (Vilsbøll *et al.*, 2001). Therefore our observation that 72 h MSG exposure leads to a decline in GLP-1 secretion suggests that it may be a contributing factor in the pathogenesis of diabetes/obesity.

A novel HCA assay measuring nuclear and mitochondrial parameters was developed to assess the cellular health of EE cells under conditions of both 3 h and 72 h MSG exposure. No cytotoxicity was evident when the EE cells were exposed to MSG for 3 h, however, significant toxic effects were observed following 72 h exposure. Concentration-dependent reductions in cell number and in mitochondrial membrane potential were observed, as were increases in nuclear area and mitochondrial mass.

MSG exposure studies are scarce in the literature. However, several *in vivo* single-dose studies have shown the potential of MSG to impact negatively upon cells and ultimately lead to cell death. One study investigated the exposure of rat thymocytes to MSG (4 mg/g body weight) and found a dose-dependent decrease in cell survival with cell loss occurring primarily through apoptosis (Pavlovic *et al.*, 2009). Increased apoptosis can arise from the absence of survival factors and cell-to-cell signalling mechanisms that are present *in vivo* (Elmore, 2007). The concentration-dependent manner in which MSG induces apoptosis has been noted previously whereby glutamate-induces cell death via apoptosis or necrosis (Ankarcrona *et al.*, 1995). The results of the present study provide no evidence of 3 h toxic effects, but again this can be due to a number of other factors including the cell type used, the MSG concentrations employed and the duration of exposure.

In the present study there was a good association between the MSG concentrations that caused decline in GLP-1 secretion and those which caused cytotoxic effects. At the highest MSG concentration tested (500 µg/ml) there was a significant decrease in both cell number and in mitochondrial membrane potential but a significant increase in mitochondrial mass and nuclear area. It is well established that biogenesis of mitochondria can increase mitochondrial mass as a result of increased mitochondrial respiration, and that this usually corresponds with reduced mitochondrial membrane potential (O'Brien and Haskins, 2007). Mitochondria are organelles involved in the regulation of programmed cell death (apoptosis). This often occurs

when cells are damaged by disease or toxic agents (Norbury and Hickson, 2001). Due to the increase in nuclear area observed in the study, it would appear that some cells underwent necrosis. In the case of necrosis, the cells will swell and consequently cause an increase in nuclear area, as observed in the present study. Although there are distinct differences in the mechanisms of apoptosis and necrosis, there is overlap between the two processes described as the “apoptosis-necrosis continuum” (Zeiss, 2003) and the results of the HCA assays suggest that the highest concentration (500 µg/ml) of MSG is capable of inducing “apoptosis-necrosis continuum” in EE cells during the 72 h exposure.

The concentrations of MSG used in this study were based on dietary exposure levels, average daily intakes across different populations and at levels higher than these levels to account for underestimations in MSG intake (Collision *et al.*, 2009; He *et al.*, 2011; Nakanishi *et al.*, 2008; Shi *et al.*, 2014). Maximum MSG solubility was achieved at 500 mg/ml, giving a top concentration on the assay plate of 500 µg/ml, equivalent to an exposure of 30 g/day. Although this is much higher than average daily intakes discussed, as outlined in the introduction, restaurants add unknown amounts of MSG to foods, therefore it is difficult to estimate accurate exposure and it is therefore possible certain groups of the population could be exposed to these high levels, where the present study found significant effects. Furthermore, in a society where MSG is not regulated and consumers are increasingly eating out or purchasing take-away foods, it is not unreasonable to suggest that MSG exposure is likely to be on the increase. It is also worth mentioning the potentially greater health risks which Asian populations are being subjected to as a result of their excessive MSG exposure. Large scale epidemiological studies are clearly warranted to provide better estimates of MSG consumption. The setting of maximum levels of MSG content in foods is something which should be given serious consideration. This would have the added benefit of improving the accuracy of consumer exposure for this food additive.

Conclusion

This is the first study to examine a potential endocrine disrupting effect of MSG, a suspected obesogen, on the interaction with the GLP-1 hormone. This study demonstrates that MSG, has the ability to stimulate GLP-1 secretion from pGIP/neo: STC-1 cells, an EE cell model following 3 h exposure, but that longer-term exposure leads to impaired GLP-1 secretion and a range of changes indicative of cytotoxicity. The multi-parameter HCA cytotoxicity assay

utilised here was able to detect subtle changes in cell health and this has great potential for investigating the potential endocrine disrupting effects of other putative obesogens.

Conflict of interest

There is no conflict of interest.

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Figure legends

Fig. 1: Images illustrating the GLP-1R redistribution. A) DMSO control and B) treated with agonist GLP-1 (300 nM). Typical HCA micrographs are shown with nuclei (blue) and GLP-1R fluorescence (green) X 20 objective magnification. 1B shows GLP-1R-EGFP internalisation that is detected by the image analysis algorithm.

Fig. 2. GLP-1 secretory responses of pGIP/neo: STC-1 cells during acute (3 h) exposure to MSG. Graph shows GLP-1 secretion from pGIP/neo: STC-1 cells (mean \pm SEM, n = 3) following 3 h incubation with 0.5-500 μ g/ml MSG.

Fig. 3. GLP-1 secretory responses of pGIP/neo: STC-1 cells during chronic (72 h) exposure to MSG. Graph shows GLP-1 secretion from pGIP/neo: STC-1 cells (mean \pm SEM, n = 3) following 72 h incubation with 0.5-500 μ g/ml MSG. $P < 0.05$ () represent significance.*

Fig. 4: Cytotoxic effects on pGIP/neo STC-1 cells of MSG 0.5- 500 μ g/ml following 72 h exposure. A number of endpoints were measured including a) Cell number, b) Nuclear area, c) Nuclear Intensity, d) Mitochondrial mass and e) Mitochondrial membrane potential. Data are expressed as a percentage of solvent control (dH₂O) for each parameter. Data are mean \pm SEM, n=3; $P < 0.05$ () < 0.01 (**) < 0.001 (***) versus appropriate control.*

Fig. 5: HCA images for a) solvent control and b) MSG-treated (500 μ g/ml) following 72 h exposure of pGIP/neo: STC-1 cells. Each image was acquired at $\times 10$ objective magnification using Hoechst dye (blue; nuclear staining) and mitochondrial potential dye (red; mitochondrial staining).

Fig. 6: GLP-1 standard curve (1-300 nM) relative to the DMSO negative control in the U2OS-GLP1R-ERFP cell line. Data are mean \pm sem, n=3. GLP-1R internalisation was normalised to vehicle control (0.2% DMSO) and 300 nM GLP-1 (100%). The EC₅₀ value was calculated at 37 nM.

643 *Figure 7: Images illustrating the GLP-1R redistribution assay following MSG exposure. A)*
644 *DMSO control, B) treated with MSG 500 $\mu\text{g/ml}$ C) Positive control (GLP-1 150 nM) and D)*
645 *MSG 500 $\mu\text{g/ml}$ and GLP-1 150 nM. Typical HCA micrographs are shown with nuclei (blue)*
646 *and GLP-1R fluorescence (green) X 20 objective magnification.*